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NEW DNA SEQUENCES AND A PROCESS FOR ENZYME MANUFACTURE

The present invention relates to an isolated or recombinant DNA sequence coding for a glucuronyl C5-epimerase capable of converting D-glucuronic acid to L-iduronic acid. The invention also relates to a process for the manufacture of such epimerase.

Background of the invention

Heparin and heparan sulfate are complex, sulfated glycosaminoglycans composed of alternating glucosamine and hexuronic acid residues. The two polysaccharides are structurally related but differ in composition, such that heparin is more heavily sulfated and shows a higher ratio of L-iduronic acid (IdoA)/D-glucuronic acid (GlcA) units (Kjellén, L. and Lindahl, U. (1991) Annual Review of Biochemistry **60**, 443-475; Salmivirta, M., Lidholt, K. and Lindahl, U. (1996) The FASEB Journal **10**, 1270-1279). Heparin is mainly produced by connective tissue-type mast cells, whereas heparan sulfate has a ubiquitous distribution and appears to be expressed by most cell types. The biological roles of heparin and heparan sulfate are presumably largely due to interactions of the polysaccharides with proteins, such as enzymes, enzyme inhibitors, extracellular-matrix proteins, growth factors/cytokines and others (Salmivirta, M., Lidholt, K. and Lindahl, U. (1996) The FASEB Journal **10**, 1270-1279). The ineractions tend to be more or less selective/specific with regard to carbohydrate structure, and thus depend on the amounts and distribution of the various sulfate groups and hexuronic acid units. Notably, IdoA units are believed to generally promote binding of heparin and heparan sulfate chains to proteins, due to the marked conformational flexibility of these residues (Casu, B., Petitou, M., Provasoli, M. and Sinay, P. (1988) Trends in Biochemical Sciences **13**, 221-225).

Heparin and heparan sulfate are synthesized as proteoglycans. The process is initiated by glycosylation reactions that generate saccharide sequences composed of alternating GlcA and N-acetylglucosamine (GlcNAc) units covalently bound to peptide core structures. The resulting (GlcAβ1,4-GlcNAcα1,4-)$_n$ disaccharide repeats are modified, probably along with chain elongation, by a series of enzymatic reactions that is initiated by N-deacetylation and N-sulfation of GlcNAc units, continues through C-5 epimerization of GlcA to IdoA residues, and is concluded by the incorporation of O-sulfate groups at various positions. The N-deacetylation/N-sulfation step has a key role in determining the overall extent of modification of the polymer chain, since the GlcA C-5 epimerase as well as the various O-sulfotransferases all depend on the presence of N-sulfate groups for substrate recognition. While the GlcNAc N-deacetylation and N-sulfation reactions are both catalyzed by the same protein, isolation and molecular cloning of N-deacetylase/N-sulfotransferase from different tissue sources implicated two distinct forms of the enzyme. The two enzyme types differ with regard to kinetic properties, and it has been suggested that they may be differentially involved in the biosynthesis of heparin and heparan sulfate.

Summary of the invention

The present invention provides for an isolated or recombinant DNA-sequence coding for a mammalian, including human, glucuronyl C-5 epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA).

The invention also provides for a recombinant expression vector containing a transcription unit comprising a DNA sequence as described above, a transcriptional promoter, and a polyadenylation sequence.

The invention also provides for a process for the manufacture of a glucuronyl C-5 epimerase or a functional

derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), comprising cultivation of a cell line transformed with the above recombinant expression vector in a nutrient medium allowing expression and secretion of said epimerase or functional derivative thereof.

Specific DNA sequences according to the invention are defined in appended claims 2, 3 and 4.

Furthermore, the invention provides for a host cell transformed with such recombinant expression vector.

Finally, the invention covers a glucuronyl C-5 epimerase or a functional derivative thereof whenever prepared by the process outlined above.

Brief description of the appended figures

Fig 1. Nucleotide sequence and the predicted amino acid sequence of the C5-epimerase. The predicted amino acid sequence is shown below the nucleotide sequence. The numbers on the right indicate the nucleotide residue and the amino acid residue in the respective sequence. The five sequenced peptides appear in **bold**. The N-terminal sequence of the purified protein is shown in ***bold and italics***. The potential N-glycosylation sites (*) are shown. The potential transmembrane region is underlined.

Fig 2. In vitro transcription-translation. The epimerase cDNA was inserted into a pcDNA3 expression vector and linearized with XbaI at the 3'-end. It was then subjected to *in vitro* transcription-translation in a rabbit reticulocyte lysate system in the presence of [^{35}S]methionine, as described in "Experimental Procedures". The translation product of epimerase cDNA (Epi) has a molecular weight of ~50 kDa, by comparison with the LMW protein standard. A 118 kDa control sample of ß-galactosidase (C), expressed in the same system, is shown for comparison.

Fig 3. Effect of the expressed C5-epimerase on N-deacetylated, N-sulfated capsular polysaccharide from E. coli K5. Metabolically ^{3}H-labeled K5 polysaccharide was N-deacetylated and N-sulfated, and was then incubated with (A) lysate of Sf9 cells infected with recombinant C5-epimerase; (B) lysate of Sf9 cells infected with recombinant ß-glucuronidase. The incubation products were treated with HNO_2 /$NaBH_4$, and the resultant hexuronyl-anhydromannitol disaccharides were recovered and separated by paper chromatography. The arrowheads indicate the migration positions of glucuronosyl-anhydromannitol (GM) and iduronosyl-anhydromannitol (IM) disaccharide standards. For further information see "Experimental Procedures".

Fig 4. Northern analysis of C5-epimerase mRNA expressed in bovine lung and mastocytoma cells. Total RNA from each tissue/cell line was separated by agarose gel electrophoresis. A blot was prepared, probed with a ^{32}P-labeled 2460-bp fragment of the epimerase cDNA clone, and finally exposed to X-ray film. (Kodak, Amersham). The arrow indicates the positions of molecular standards. For further information see "Experimental Procedures".

Detailed description of the invention

The present invention relates to DNA sequences coding for a mammalian glucuronyl C5-epimerase or a functional derivative thereof, such epimerase or derivative being capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA). The term "mammalian" is intended to include also human varieties of the enzyme.

As used herein the definition "glucuronyl C5-epimerase or a functional derivative thereof" refers to enzymes which have the capability of converting D-glucuronic acid to L-iduronic acid. Accordingly, the definition embraces all epimerases having such capability including functional variants, such as functional frag-

ments, mutants resulting from mutageneses or other recombinant techniques. Furthermore, the definition is intended to include glycosylated or unglycosylated mammalian glucuronyl C5-epimerases, polymorfic or allelic variants and other isoforms of the enzyme. "Functional derivatives" of the enzyme can include functional fragments, functional fusion proteins or functional mutant proteins. Such epimerases included in the present invention can have a deletion of one or more amino acids, such deletion being an N-terminal, C-terminal or internal deletion. Also truncated forms are envisioned as long as they have the conversion capability indicated herein.

Operable fragments, mutants or truncated forms can suitably be identified by screening. This is made possible by deletion of for example N-terminal, C-terminal or internal regions of the protein in a step-wise fashion, and the resulting derivative can be analyzed with regard to its capability of the desired conversion of D-glucuronic acid to L-iduronic acid. If the derivative in question operates in this capacity it is considered to constitute a functional derivative of the epimerase proper.

Examples of useful epimerases are proteins having the sequence as shown in Fig. 1 or substantially as shown in Fig. 1 and functional portions thereof.

EXPERIMENTAL PROCEDURES

Peptide Purification and Sequencing - The 52 kDa epimerase protein (~1µg), purified from a detergent extract of bovine liver by chromatography on O-desulfated heparin-Sepharose, Red-Sepharose, Phenyl-Sepharose, and Concanavalin A-Sepharose (Campbell, P., Hannesseon, H.H., Sandbäck, D., Rodén, L., Lindahl, U. and Li, J.-p. (1994) J Biol Chem **269**, 26953-26958), was subjected to direct N-terminal sequencing using a model 470A protein sequenator (Applied Biosystems) equipped with an on-line 120 phenylthiohydantoin analyzer (Tempst, P., and Riviere, L.

(1989) Anal. Biochem. **183**, 290-300). Another sample (~1µg) was applied to preparative (12%) SDS-PAGE and was then transferred to a PVDF membrane. After staining the membrane with Coomassie Blue, the enzyme band was excised. Half of the material was submitted to direct N-terminal sequence analysis, whereas the remainder was digested with Lys-C (0.0075 U; Waco) in the presence of 1% RTX-100/10% acetonitrile/100mM Tris-HCl, pH 8.0. The generated peptides were separated on a reverse phase C4-column, eluted at a flow rate of 100 µl/min with a 6-ml 10-70% acetonitrile gradient in 0.1% trifluoroacetic acid, and detected with a 990 Waters diode-array detector. Selected peptides were then subjected to sequence analysis as described above.

Probes for Screening - Total RNA was extracted from bovine liver according to the procedures of Sambrook *et al.* (1989). Single-stranded cDNA was synthesized by incubating ~5 µg of bovine liver total RNA (denatured at 65°C, 3 min) with a reaction mixture containing 1 unit RNAse inhibitor (Perkin-Elmer Corp.), 1 mM of each dNTP, 5 µM random nucleotide hexamer and 1.25 units of murine leukemia virus reverse transcriptase (Perkin-Elmer Corp.) in a buffer of 10 mM Tris-HCl, pH 8.3. The mixture was kept at 42°C for 45 min and then at 95°C for 5 min. Degenerated oligonucleotide primers were designed based on the amino-acid sequence determined for one of the internal peptides derived from the purified epimerase (Table I). Single-stranded bovine liver cDNA was applied to PCR together with 100 pmols of primers 1 (sense) and 3 (antisense), in a total volume of 100 µl containing 1µl of 10% Tween 20, 6 mM $MgCl_2$, 1 mM of each dNTP, and 2.5 units Taq polymerase (Pharmacia Biotech) in a buffer of 10 mM Tris-HCl, pH 9.0. The reaction products were separated on a 12% polyacrylamide gel. A ~100-bp band was cut out from the gel and reamplified using the same PCR conditions. After an additional polyacrylamide gel electrophoresis, the product was isolated and sequenced ,

yielding a 108-bp sequence. This PCR product was subcloned into a pUC119 plasmid. The DNA fragment cleaved from the plasmid was labeled with [^{32}P]dCTP (DuPont NEN) using a Random Primed DNA Labeling Kit (Boehringer Mannhem).

Screening of cDNA Library - A bovine lung cDNA library constructed in a lgt10 vector (Clontech) was screened with the 108-bp PCR fragment as hybridizing probe. The nitrocellulose replicas of the library plaques were prehybridized in 6xSSC, 5xDenhart's solution containing 0.1% SDS and 0.1 mg/ml denatured salmon DNA for 2 hours at 65°C. Hybridization was carried out at 42°C in the same solution containing ^{32}P-labled probe for 16-18 hours. The filters were washed two times with 2x SSC, 0.5% SDS and two times with 0.5x SSC, 1% SDS at the same temperature. The library was repeatedly screened twice under the same conditions. Finally, the entire cDNA phage library was subjected to PCR amplification using lgt10 forward and reverse primers (Clontech) with a epimerase cDNA specific primer (5'-GCTGATTCTTTTCTGTC-3').

Subcloning and Sequencing of cDNA Inserts - cDNA inserts, isolated by preparative agarose gel elctrophoresis (Sambrook *et al.*, 1989) after *Eco*RI restriction cleavage of recombinant bacteriophage DNA, were subcloned into a pUC119 plasmid. The complete nucleotide sequence was determined independently on both strands using the dideoxy chain termination reaction either with [^{35}S]dATP and the modified T7 DNA polymerase (Sequenase version 2.0 DNA Sequencing Kit; U. S. Biochemical Corp.) or the ALF™ System (Pharmacia Biotech). DNA sequences were compiled and analyzed using the DNASTAR™ program (Lasergene).

Polyclonal Antibodies and Immunodetection- A peptide corresponding to residues 77 - 97 of the deduced epimerase amino-acid sequence was chemically synthesized (Åke Engström, Department of Medical and Physiological Chemistry, Uppsala University, Sweden), and was then conjugated to ovalbumin using glutaraldehyde (Harlow, E. and Lane,

D. (1989) in Antibodies: A Laboratory Manual, pp 78-79, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). A rabbit was immunized with the peptide conjugates together with Freund' s adjuvant. After 6 boosts (each with 240 µg conjugated peptide) blood was collected and the serum recovered. The antibody fraction was further purified on a Protein A-Sepharose column (Pharmacia Biotech), and used for immunoblotting.

Samples of GlcA C5-epimerase were separated under denaturing conditions by 12% SDS-PAGE, and were then transferred to a nitrocellulose membrane (Hybond™ ECL). ECL immunoblotting was performed according to the protocol of the manufacturer (Amersham). Briefly, the membrane was first treated with blocking agent, then incubated with purified antibody, and finally incubated with the peroxidase labeled anti-rabbit antibody. After adding the ECL reagent, the light emitted by the chemical reaction was detected by exposure to Hyperfilm™ ECL for 30-60 sec.

Northern Blot Hybridization -- Bovine liver and lung total RNA was prepared according to Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harabor Laboratory, Cold Spring Harbor NY), and mouse matocytoma (MCT) total RNA was extracted from a tumor cell line (Montgomery, R.I., Lidholt, K., Flay, N.W., Liang, J., Verter, B., Lindahl, U. and Esko, J.D. (1992) PNAS **89**, 11327-113331) as described by Chomczynski and Sacci (1987). Total RNA from each tissue (~20 µg samples) was denatured in 50% formamide (v/v), 5% formaldehyde, 20 mM Mops buffer, pH 7.0, at 65 °C for 5 min. The denatured RNA was separated by electrophoresis in 1.2% agarose gel containing 5% formaldehyde (v/v), and was then transferred to a Hybond N⁺ nylon membrane (Amersham). The RNA blot was pre-hybridized in ExpressHyb Hybridization Solution (Clontech) at 65 °C for 1 h, and subsequently hybridized in the same solution with-a [^{32}P]dCTP-labeled DNA probe (a 2460 bp fragment including the 5'-end of the cDNA clone; see

Fig.1). The membrane was washed in 2x SSC, 0.5% SDS at the same temperature for 2 x 15 min and in 0.5x SSC, 0.5 % SDS for 2 x 15 min. The membrane was exposed to a Kodak X-ray film at -70°C for 24h.

In Vitro Translation - The 3-kb GlcA C5-epimerase clone, inserted in a pcDNA3 expression vector (Invitrogen) was linearized at the 3'-end by restriction enzyme *Xba*I. *In vitro* translation was carried out with a Linked T7 transcription-translation system (Amersham) according to the instructions of the manufacturer. The corresponding mRNA generated by incubation of 0.5 µg linearized plasmid DNA with a T7 polymerase transcription mix (total volume, 10 µl; 30°C; 15 min) was mixed with an optimized rabbit reticulocyte lysate containing 50µCi [^{35}S]methionine (total volume, 50 µl), and further incubated at 30 °C for 1 h. A sample (5 µl) of the product was subjected to 12% SDS-PAGE. The gel was directly exposed to a Kodak X-ray film. After exposure, the applied protein molecular standards (LMW Molecular Calibration Kit, Pharmacia Biotech) were visualized by staining the gel with Coomassie Blue.

Expression of the GlcA C5-Epimerase - The GlcA C5-epimerase was expressed using a BacPAK8™ Baculovirus Expression System (Clontech), according to the instructions by the manufacturer. Two oligonucleotides, one at the 5'-end of the cDNA clone (1-17 bp, sense) and the other at the 3'-end of the coding sequence (1387-1404 bp, antisense), were used to PCR amplify the coding sequence of the C5-epimerase cDNA clone. The resulting fragment was cloned into the BacPAK8 vector. Sf9 insect cells, maintained in Grece's Insect Medium (GibcoBRL) supplemented with 10% fetal calf serum and penicillin/streptomycin, were then cotransfected by the C5-epimerase construct along with viral DNA. Control transfections were performed with constructs of a ß-glucuronidase cDNA construct included in the expression kit, and a mouse cDNA coding for the GlcNAc N-deacetylase/N-sulfotransferase

implicated in heparin biosynthesis (Eriksson, I., Sandbäck, D., Ek, B., Lindahl, U. and Kjellén, K. (1994) J.Biol. Chem. **269**, 10438-10443; Cheung, WF., Eriksson, I., Kusche-Gullberg, M., Lindahl, U. and Kjellén, L. (1996) Biochemistry **35**, 5250-5256). Single plaques of each co-transfected recombinant were picked and propagated. Two Petri dishes (60-mm) of Sf9 cells were infected by each recombinant virus stock and incubated at 27°C for 5 days. The cells from one dish were used for total RNA extraction and Northern analysis performed as described above. Cells from the other dish were lysed in a buffer of 100 mM KCl, 15 mM EDTA, 1% Triton X-100, 50 mM HEPES, pH 7.4, containing 1mM PMSF and 10µg/ml pepstatin A. Supernatants of cell lysates as well as conditioned media were analyzed for epimerase activity. Protein contents of the cell lysates were estimated by the method of Bradford (1976) or by the BCA reagent procedure (Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985) Anal. biochem **150, 76-85)**.

Demonstration of GlcA C5-epimerase activity - Epimerase activity was assayed using a biphasic liquid scintillation counting procedure, essentially as described by Campbell *et al.* (1994) above. The reaction mixtures, total volume 55 µl, contained 25 µl cell lysate or medium, 25 µl of 2x epimerase assay buffer (20 mM HEPES, 30 mM EDTA, 0.02% Triton X-100, 200 mM KCl, pH 7.4) and 5 µl of substrate (10,000 cpm ^{3}H). The substrate was a chemically N-deacetylated and N-sulfated polysaccharide, obtained from *E. coli* K5 according to the procedure of Campbell *et al.* (1994), except that D-[5-^{3}H]glucose was substituted for D-[1-^{3}H]glucose.

Enzymatic conversion of D-glucuronic to L-iduronic acid was demonstrated using the metabolically 1-^{3}H-labeled substrate (N-deacetylated, N-sulfated capsular polysaccharide from *E. coli* K5) and the analytical proce-

dure described by Campbell *et al.* (1994). A sample (~20 µg; 200,000 cpm of ^3H) of the modified polymer was incubated with 250 µl of cell lysate in a total volume of 300 µl epimerase assay buffer at 37°C for 6 hours. The incubation was terminated by heating at 100°C for 5 min. The sample was mixed with 50µg of carrier heparin and reacted with nitrous acid at pH 1.5 (Shively, J., and Conrad, H.E. (1976) Biochemistry **15**, 3932-3942), followed by reduction of the products with $NaBH_4$. The resultant hexuronyl-anhydromannitol disaccharides were recovered by gel chromatography on a column (1 x 200 cm) of Sephadex G-15 in 0.2 M NH_4HCO_3, lyophilized, and subjected to paper chromatography on Whatman No 3MM paper in ethyl acetate/acetic acid/water (3:1:1).

RESULTS

Generation of a Probe and Screening of cDNA Library - Amino acid sequence data for the ~52 kDa protein were obtained by digesting highly purified epimerase with lysine-specific protease, followed by separation of the generated peptides on a reverse phase column. The five most prominent peptides were isolated and subjected to amino-acid sequencing (Table I). One of the peptides (peptide 1) was found to correspond to the N-terminal sequence of the native protein. The sequence of the largest peptide obtained (peptide 5 in Table I), was used to design two sense and one antisense degenerate oligonucleotide primers, as shown in Table I. A DNA probe was produced by PCR using primers 1 and 3 with bovine liver cDNA as template. The resultant ~100 bp DNA fragment was purified by polyacrylamide gel electrophoresis, reamplified using the same primers, and finally isolated by electrophoresis. The identity of the product was ascertained by "nested" PCR, using primers 2 and 3, which yielded the expected ~60 bp fragment (data not shown). Moreover, sequencing of the larger (108 bp) DNA fragment gave a de-

duced amino-acid sequence identical to that of the isolated peptide (Table I).

The 108-bp PCR product was labeled with [^{32}P]dCTP and used for screening of a bovine lung lgt10 library. One hybridizing clone, containing a 3-kb insert, was identified. Repeated screening of the same library yielded two additional positive clones, both of which were of smaller size. Subsequent sequencing showed both of the latter clones to be contained within the 3.0-kb species (data not shown). The 3-kb clone was sequenced through both strands and was found to contain altogether 3073 bp; an additional 12-bp sequence was added at the 5'-end through characterization of a separate clone obtained by PCR amplification of the phage library (see "Experimental Procedures").

Characterization of cDNA and Predicted Protein Structure - The total cDNA sequence identified, in all 3085 bp, contains an open reading frame corresponding to 444 amino-acid residues (Fig. 1). Notably, the coding region (1332 bp) is heavily shifted toward the 5'-end of the available cDNA, and is flanked toward the 3'-end by a larger (1681 bp) noncoding segment. The deduced amino-acid sequence corresponds to a 49,905 dalton polypeptide. All of the five peptides isolated after endo-peptidase digestion (Table I) were recognized in the primary structure deduced from the cDNA (Fig. 1). One of these peptides (peptide 1) is identical to the N-terminus of the isolated liver protein. This peptide was found to match residues 74 - 86 of the deduced polypeptide sequence. The enzyme isolated from bovine liver thus represents a truncated form of the native protein.

Generation of mRNA from an expression vector inserted with the 3-kb cDNA clone, followed by incubation of the product with rabbit reticulocyte lysate in the presence of [^{35}S]methionine, resulted in the formation of a distinct labeled protein with an estimated M_r of ~50kDa (Fig. 2). This product was recognized in immunoblotting

(data not shown) by polyclonal antibodies raised against a synthetic peptide corresponding to residues 77 - 97 (see Fig. 1) of the deduced amino-acid sequence. The same antibodies also reacted with the isolated ~52 kDa bovine liver protein (data not shown). These observations establish that the 3-kb cDNA is derived from the transcript that encodes the isolated ~52 kDa bovine liver protein.

The cDNA structure indicates the occurrence of 3 potential N-glycosylation sites (Fig.1). Sugar substituents may be important for the proper folding and catalytic activity of the enzyme, since the protein expressed in bacteria (which also gave a strong Western signal towards the polyclonal antibodies raised against the synthetic peptide; data not shown) was devoid of enzymatic activity. A potential transmembrane region is underlined in Fig. 1. The predicted protein contains two cystein residues, only one of which occurs in the isolated (truncated) protein. Since NEM was inhibitory to epimerase activity (data not shown), this single cystein unit may be essential to the catalytic mechanism.

Functional Expression of the GlcA C5-Epimerase - A variety of expression systems were tested in attempts at generating the cloned protein in catalytically active form. A protein *obtained* by in vitro translation using a rabbit reticulocyte lysate system (see Fig. 2) showed no detectable epimerase activity. A construct made by inserting the 3-kb cDNA into a pcDNA3 vector (Invitrogen) failed to induce mRNA formation (or translation) in any of the cell lines tested (human embryonic kidney (293), COS-1 or CHO cells) (data not shown). We also attempted to express the enzyme in a bacterial pET system (Novagen). The transformed bacteria yielded appreciable amounts of immunoreactive protein which, however, lacked detectable enzyme activity (data not shown).

Cotransfection of epimerase recombinant with baculovirus into Sf9 insect cells resulted in the generation of abundant GlcA C5-epimerase activity (Table II). In two

separate experiments, the lysates from cells infected with the same epimerase recombinant virus stock showed >10-fold higher enzyme activities, on a mg protein basis, than the corresponding fractions from cells infected with control recombinant virus stock. The conditioned media of cells infected with epimerase recombinant showed 20- 30-fold higher enzyme activities than the corresponding fractions from cells infected with control plasmid virus stock. Transfections with cDNA encoding other enzymes, such as a ß-glucuronidase, or the mouse mastocytoma GlcNAc N-deacetylase/N-sulfotransferase involved in heparin biosynthesis (Eriksson *et al.*, 1994), did not significantly increase the epimerase activity beyond control levels. Notably, the higher ^3H_2O release recorded for control samples as compared to heat-inactivated expressed enzyme (Table II) suggests that the insect cells constitutively produce endogenous C5-epimerase.

The polysaccharide substrate used for routine assays of epimerase activity was obtained by chemically N-deacetylating and N-sulfating the capsular polysaccharide [(GlcAß1,4-GlcNAcα1,4)n] of E. coli K5 that had been grown in the presence of [5-^{3}H]glucose. The data in Table II thus reflect the release of ^3H_2O from 5-^{3}H-labeled GlcA units in the modified polysaccharide, due to enzyme action (Jacobsson, I., Bäckström, G., Höök, M., Lindahl, U., Feingold, D.S., Malmstrom, M, and Rodén, L. (1979) J.Biol. Chem. 254, 2975-2982; Jacobsson,I., Lindahl, U., Jensen, J.W., Rodén, L., Prihar, H. and Feingold, D.S. (1984) Journal of Biological Chemistry **259**, 1056-1064). More direct evidence for the actual conversion of GlcA to IdoA residues was obtained by incubating the expressed enzyme with an analogous substrate, obtained following incubation of the bacteria with [1-^{3}H]glucose. This substrate will retain the label through the epimerization reaction, and can therefore be used to demonstrate the formation of IdoA-containing disaccharide units. Following incubation with the recombinant epimerase, 21% of the

hexuronic acid residues was converted to IdoA, as demonstrated by paper chromatography of disaccharide deamination products (Fig. 3). The composition of the incubated polysaccharide thus approached the equilibrium ratio of IdoA/GlcA, previously determined to ~3/7[1].

Northern Analysis -Total RNA, from bovine liver, lung, and mouse mastocytoma, were analysed by hybridization with a 2460-bp DNA fragment from epimerase cDNA clone as a probe. Both bovine liver and lung gave identical transcription patterns, with a dominant transcript of ~9 kb and a weak ~5 kb band (Fig. 4). By contrast, the mastocytoma RNA showed only the ~5 kb transcript.

It is to be noted that the present invention is not restricted to the specific embodiments of the invention as described herein. The skilled artisan will easily recognize equivalent embodiments and such equivalents are intended to be encompassed in the scope of the appended claims.

CLAIMS

1. An isolated or recombinant DNA sequence coding for a mammalian, including human, glucuronyl C5-epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA).

2. A DNA sequence according to claim 1 constituted by a nucleotide sequence comprising nucleotide residues 1 to 1404, inclusive, as depicted in Fig. 1.

3. A DNA sequence according to claim 2 constituted by a nucleotide residue comprising nucleotide residues 73 to 1404, inclusive, as depicted in Fig. 1.

4. A DNA sequence according to claim 2 constituted by a nucleotide residue comprising nucleotide residues 1 to 1404, inclusive, as depicted in Fig. 1.

5. A recombinant expression vector containing a transcription unit comprising a DNA sequence according to any one of the preceding claims, a transcriptional promoter, and a polyadenylation sequence.

6. A host cell transformed with the recombinant expression vector of claim 5.

7. A process for the manufacture of a glucuronyl C5-epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA), comprising cultivation of a cell line transformed with a recombinant expression vector according to claim 5 in a nutrient medium allowing expression and secretion of said epimerase or functional derivative thereof.

8. A glucuronyl C5-epimerase or a functional derivative thereof whenever prepared by the process of claim 7.

ABSTRACT

An isolated or recombinant DNA sequence coding for a mammalian, including human, glucuronyl C5-epimerase or a functional derivative thereof capable of converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA);

a recombinant expression vector comprising such DNA sequence;

a host cell transformed with such recombinant expression vector;

a process for the manufacture of a glucuronyl C5-epimerase of functional derivative thereof capable of converting GlcA to IdoA, comprising cultivation of a cell-line transformed with such recombinant expression vector; and

a glucuronyl C5-epimerase or functional derivative thereof prepared by such process.

Table I
Peptide and primer sequences

A. N-terminal sequences of isolated C5-epimerase
1. PNDWXVPKGCFMA (free solution)
2. PXDWTVPKGXF (band excised from PVDF-membrane)

B. Peptide sequences
1. PNDXTVPK
2. XXIAPETSEGXSLQL
3. GGWPIMVTRK
4. FLSEQHGV
5. KAMLPLYDTGSGTIYDLRHFMLGIAPNLAXWDYHTT

	primer 1 (sense)	primer 2 (sense)	primer 3 (antisense)

C. Primer[a]		Degeneracy
1 (S)	5'-cc gaattcAARGCNATGYTNCCNYT-3'[b]	384
2 (S)	5'-cc gaattcGAYYTNMGNCAYTTYATG-3'	288
3 (AS)	5'-cc ggatccGTNGTRTGRTARTCCCA-3'	32

[a] (R, A or G; Y, T or C; M, C or A; N, A or C or G or T)
[b] (cc, clamp; gaatcc, EcoRI restriction site; ggatcc, BamHI restriction site)

Table II

Expression of HexA C5-epimerase in Sf9 cells

Sf9 cells ($1x10^6$ in 4 ml medium) were seeded in 60-mm Petri dishes and incubated for three hours at 27°C. After the cells were attached, the medium was removed, and 200 µl of recombinant virus stock was added to infect the cells at room temperature for 1h. The virus suspension was aspirated and 4 ml of medium was added to each dish. The cells were incubated at 27 °C for 5 days. The medium was transferred into a steril tube and centrifuged. The cells were collected, washed twice with PBS and lysed with 300 µl of homogenization buffer as described under "Experimental Procedures". Aliquots (25 µl) of cell lysate and medium were assayed for epimerase activity. The activity is expressed as release of ^3H from K5 polysaccharide per hour. The data is mean value of three independent assays.

Construct	Epimerase Activity	
	Cell lysate (cpm/mg/h)	Medium (cpm/ml/h)
HexA C5-Epimerase-1	102670 ± 5540	45200 ± 1770
HexA C5-Epimerase-2	123270 ± 4660	52610 ± 810
HexA C5-Epimerase-1 (heat-inactivted)	240	610
N-Deacetylase/sulfotransferase	9520 ± 620	1350 ± 280
ß-Glucuronidase	8460 ± 1270	1610 ± 440
BacPAK plasmid	5150 ± 880	2820 ± 690
Neo	7250 ± 370	550 ± 120

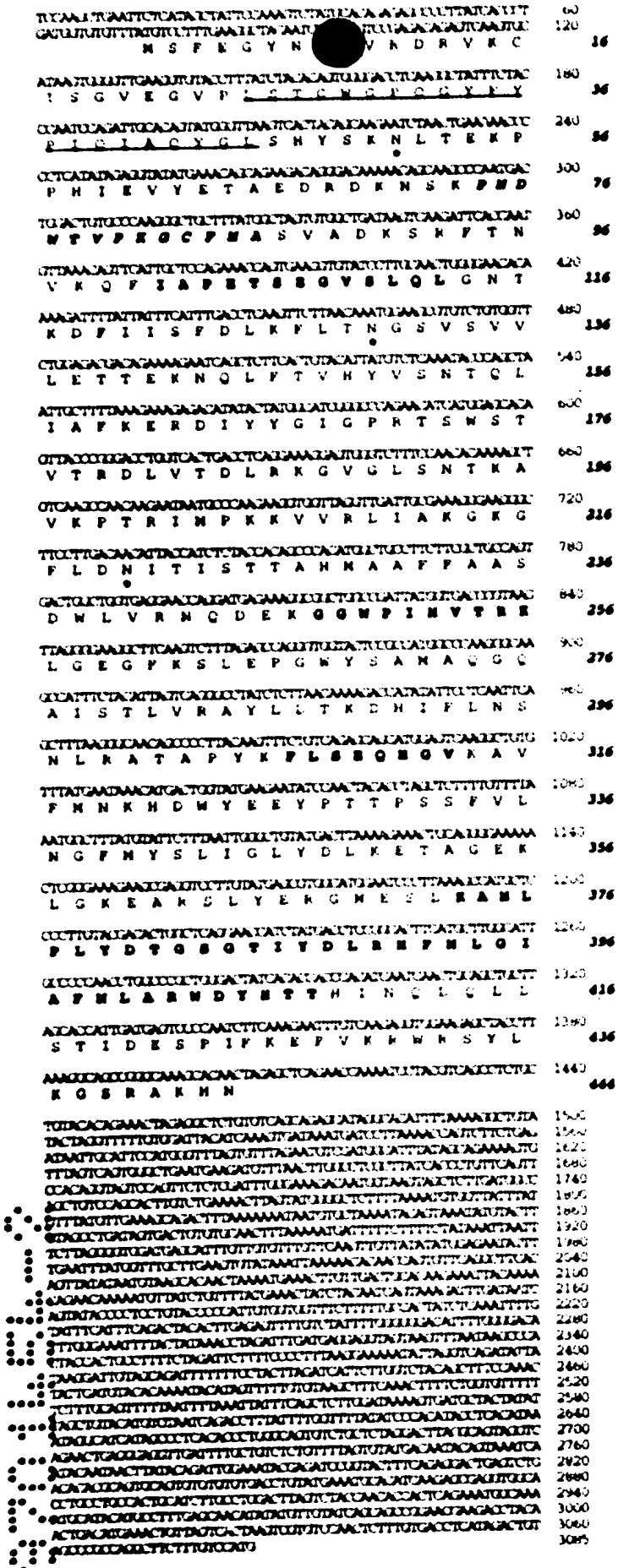

FIG. 1

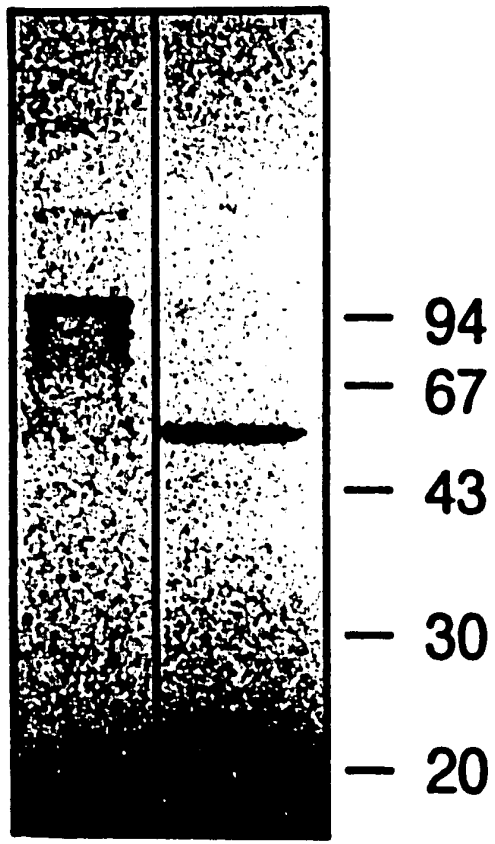

FIG. 2

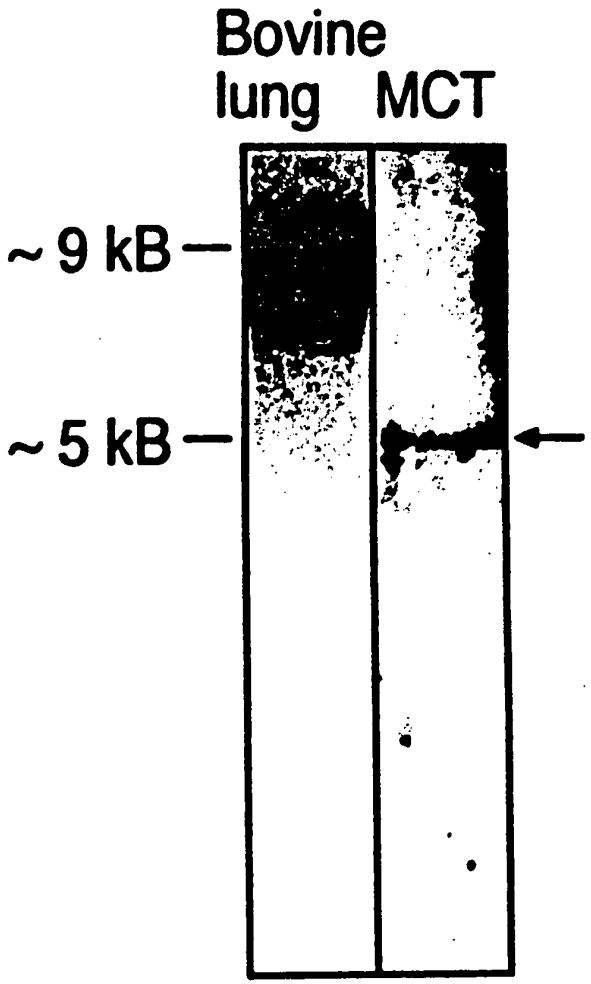

FIG. 4

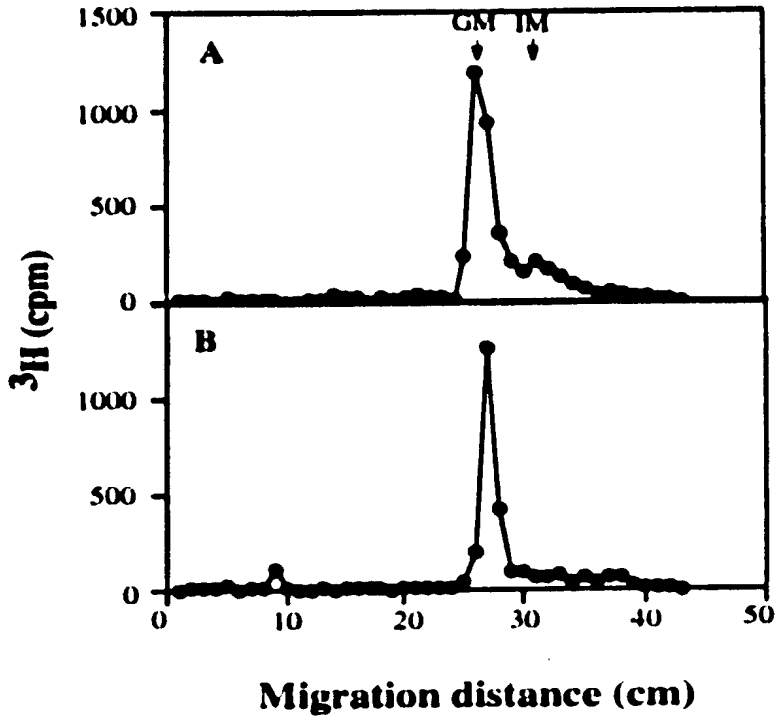

FIG. 3